De novo generation of antigen-specific $CD4^+CD25^+$ regulatory T cells from human $CD4^+CD25^-$ cells

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Antigen-specificity is a hallmark of adaptive T cell-mediated immune responses. $CD4^+CD25^+FOXP3^+$ regulatory T cells (T_R) also require activation through the T cell receptor for function. Although these cells require antigen-specific activation, they are generally able to suppress bystander T cell responses once activated. This raises the possibility that antigen-specific T_R may be useful therapeutically by localizing generalized suppressive activity to tissues expressing select target antigens. Here, we demonstrate that T_R specific for particular peptide-MHC complexes can be generated from human $CD4^+CD25^-$ T cells *in vitro* and isolated by using HLA class II tetramers. Influenza hemagglutinin epitopes were used to generate hemagglutinin-specific T_R, which required cognate antigen for activation but which subsequently suppressed noncognate bystander T cell responses as well. These findings have implications for the generation of therapeutic regulatory T cells in disease, and also suggest an important mechanism by which T cells may be regulated at the site of inflammation.

autoimmunity | T lymphocytes | tolerance | suppression | anergy

The immune system has evolved a series of mechanisms to protect against autoimmunity or excessive inflammatory responses to pathogens. It has become increasingly clear that $CD4^+CD25^+$ regulatory T cells (T_R) are an important component of this immune regulation in the periphery. In both humans and mice, $CD4^+CD25^+$ T_R have been shown to suppress T cell responses in a contact-dependent, cytokine-independent manner and to require activation via the T cell receptor to be functional. The *forkhead*-family transcription factor FoxP3 is essential for the development and function of $CD4^+CD25^+$ T_R, and spontaneous mutation of FoxP3 leads to widespread lymphocytosis and autoimmunity in the *scurfy* mouse and in humans with immune disregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (1, 2). Studies in animal models of autoimmunity have also shown an increased frequency or severity of autoimmunity in the absence of T_R (3–7) and that transfer of T_R is sufficient to protect from or reverse autoimmunity.

Recent studies have suggested that T_R may be antigen-specific. Freshly isolated unmodified $CD4^+CD25^+$ T cells have been able to suppress proliferation in assays using peptides to stimulate cells rather than polyclonal anti-CD3 stimulation (7, 8). Clones have also been generated that express CD25 and are suppressive when given cognate antigen (9, 10). Alloantigen stimulation of both human and mice $CD4^+CD25^+$ T cells (11, 12) or priming mice with alloantigen (13) has also resulted in antigen-specific T_R. The induction of T_R has now been described in both mouse and man. T_R have been induced *in vivo* in mice by administration of oral or i.v. antigen (14), antigen emulsified in incomplete Freund's adjuvant (15), retroviral delivery of an autoantigen–IgG fusion construct (16), or repeated exposure to superantigen (17). In humans, tumor lysate-loaded antigen presenting cells (18) and epitope-specific immunotherapy (19) have been shown to increase the number of circulating T_R. Studies of infection with leishmania (20) have demonstrated the protective effect of these regulatory cells in the response to inflammation directed against foreign antigens, as well. Recently, $CD4^+CD25^+$ T_R, which express FoxP3, have been induced *in vitro* in mice by activation of $CD4^+CD25^-$ T cells in the presence of TGF-β (21, 22). *In vivo* studies have shown that transferred $CD4^+CD25^-$FoxP3- T cells can differentiate into T_R (23). In addition, studies in animal models of autoimmunity have demonstrated the therapeutic benefit of transfer of antigen-specific T_R (24, 25). These data suggest that T_R are not uniquely specific for self-antigens, and that those with the potential to regulate responses to foreign antigens are either expanded upon stimulation with cognate antigen, or are generated *de novo* in the periphery during the response to that antigen.

The ability to isolate human antigen-specific $CD4^+CD25^+T_R$ holds the promise of an immunosuppressive therapy targeted to specific tissues. However, the isolation of T_R from the peripheral blood is difficult. $CD4^+CD25^{high}$ cells represent only 3% of $CD4^+$ T cells in the blood, and the precursor frequency of CD4 T cells to any specific peptide can range from 1 in 2,000 to 1 in 200,000 or greater (26). Here, we take an alternative approach to solve the problem of isolating antigen-specific T_R. We have recently shown that $CD4^+CD25^+$ T cells with regulatory activity can be generated *ex vivo* from previously nonregulatory $CD4^+CD25^-$ T cells (27). These *in vitro* generated T_R share the characteristics of $CD4^+CD25^+$ T_R taken directly from the peripheral blood, including the expression of FoxP3, and an ability to suppress in a cell-contact-dependent, TGF-β- and IL-10-independent manner. Here, we extend these findings to demonstrate that $CD4^+$ T cells from both the naïve and memory cell compartments can be induced to become T_R. We further show that the *de novo* generation of T_R can be accomplished under a variety of culture conditions including exposure to antigen-presenting cells (APCs) and antigen, demonstrating that triggering through an antigen-specific T cell receptor with a specific peptide–MHC complex can induce T_R *in vitro*. We also show that regulatory functions of these cells requires antigen-specific triggering, indicating an important mechanism for control of T cell immunity and suggesting opportunities for therapeutic manipulation.

Methods

Clinical Samples. Blood samples used here were obtained from healthy volunteers participating in a research protocol approved by the institutional review board. The HLA class II type of all participants had been previously obtained, and samples used for hemagglutinin (HA) experiments were from immunized individuals.

Derivation and Culture of Mature DC. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation over Ficoll-Hypaque gradients. Cells were plated for adherence for 2 h and

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Abbreviations: T_R, regulatory T cell; HA, hemagglutinin; Tmr, tetramer; APC, antigen-presenting cell.

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then washed of nonadherent cells. Cells were cultured with 1,000 units/ml granulocyte/macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml IL-4 (R & D Systems). After 6–9 days, 2 ng/ml IL-1β (R & D Systems), 10 ng/ml TNF-α (R & D Systems), 5 units/ml IL-6 (R & D Systems), and 1 μg/ml PGE_2 (Calbiochem) were added to the culture to mature cells for 2–3 days. Cells were then harvested, irradiated (5,000 rads), and used to stimulate $CD4^+CD25^-$ cells.

Generation of Regulatory T Cells. To isolate $CD4^+CD25^-$ T cells, PBMC were prepared by centrifugation over Ficoll–Hypaque gradients. $CD4^+$ T cells were purified by depletion of cells expressing CD8, CD11b, CD16, CD19, CD36, and CD56 with the $CD4^+$ No-touch T cell isolation kit (Miltenyi Biotec). $CD25^-$ cells were isolated by negative selection with CD25 microbeads (Miltenyi Biotec). Purity was determined to be >99% $CD25^-$, and cells were activated with either allogeneic DC, 10 μg/ml HA (306–319, PKYVKQNTLKLAT), and irradiated (5,000 rads) CD4 depleted PBMC, or 5 μg/ml plate-bound anti-CD3 (UCHT1) and 1 μg/ml soluble anti-CD28. Cells were removed from the plate-bound antibody after 24 h. After 10 days of culture, cells were sorted based on expression of CD25, CD4, and tetramer (Tmr).

Suppression of Proliferation by $CD25^+$ Cells. For the suppression assay, $CD4^+CD25^-$ cells (25,000 cells per well), $CD4^+CD25^+$ cells (25,000 cells per well), or both (25,000 cells per well each) were activated with either 10 μg/ml HA (306–319), and/or 10 μg/ml tetanus toxoid (Wyeth-Ayerst), or 5 μg/ml each soluble anti-CD3 and anti-CD28 along with T cell-depleted accessory cells (100,000 cells per well). Proliferation was measured by adding 1 μCi of [^{3}H]thymidine during the final 16 h of a 5- to 6-day assay. For transwell experiments, cells were cultured in 24-well plates with or without a 4-μm transwell separating $CD4^+CD25^+$ (50,000 cells per well) cells from $CD4^+CD25^-$ (50,000 cells per well). To test the dependence of suppression on cytokines, 10 μg/ml anti-IL-10 (JES3-19F1, Pharmingen), anti-TGF-$\beta_{1,2,3}$ (1D11, R & D Systems) or isotype-matched controls (R35–95, MOPC-21, Pharmingen) were added to the suppression assay.

Analysis of FoxP3 Expression. Isolated T cells were washed in PBS then lysed and sonicated in lysis buffer (25 mM Tris, pH 8.5/2% lithium dodecyl sulfate/1 mM EDTA/10 mM sodium fluoride/1 mM sodium orthovanadate/1× Roche Complete protease inhibitors) and quantified by BCA (Pierce). Lysates were separated on 4–12% gradient bis-Tris gels (Invitrogen) and transferred to nitrocellulose. Membranes were blocked for 3 h in TBS/0.1% Tween 20 with 5% nonfat dry milk, probed with polyclonal rabbit-anti-FoxP3 antiserum (1:2,000) overnight at 4°C, and developed as described (28). For loading control, blots were stripped and reprobed for TFIIB (Santa Cruz Biotechnology). Positive control lysate was from 293T cells transfected with human FoxP3 cDNA.

Preparation of HLA-DR0401 Tetramers. The construction of the expression vectors for generation of the soluble DRA*0101/DRB1*0401 has been described (29). Briefly, a site-specific biotinylation sequence was added to the 3′ end of the DRB1*0401 leucine zipper cassette, and the chimeric cDNA was subcloned into a Cu-inducible *Drosophila* expression vector. DR-A and DR-B expression vectors were cotransfected into Schneider S-2 cells, purified, concentrated, and biotinylated. Specific peptide was loaded for 48–72 h, and tetramers were formed by incubating class II molecules with phycoerythrin-labeled streptavidin. For staining with tetramers, cells were incubated for 1 h at 37°C with 50 μg/ml tetramer.

Results

$CD4^+CD25^+$ T_R Can Be Generated from Either Naïve or Memory T Cells. Freshly isolated $CD4^+CD25^+$ T_R from peripheral blood express the cell surface marker CD45RO and have shortened telomeres consistent with a memory phenotype, raising the possibility that T_R derive from the memory pool (8). Thus, we examined the ability of naïve and memory peripheral blood $CD4^+$ T cells to differentiate into T_R *in vitro*. $CD4^+$ T cells from peripheral blood were divided into naïve and memory pools based on expression of the markers CD45RO or CD45RA. $CD4^+CD25^-CD45RA^+CD45RO^-$ (naïve), or $CD4^+CD25^-$CD45RA-$CD45RO^+$ (memory) populations were isolated by FACS and placed in culture under conditions shown to generate T_R (27). After 10 days of culture, cells were sorted on the basis of CD4 and CD25 expression and assayed for T_R function in a suppression assay (Fig. 1). As previously shown, the $CD25^-$ cells used in these generation cultures have a purity of >99% and can be FACS sorted with a purity of >99.9%, making it unlikely that the $CD25^+$ cells obtained after 10 days of culture are the result of an expansion of contaminating $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ cells from each starting population were capable of suppressing freshly isolated $CD25^-$ T cells in a cell-contact-dependent (Fig. 1*a*), TGF-β- and IL-10-independent (Fig. 1*b*) manner, whereas $CD25^-$ cells isolated from the same cultures did not. The relative ability of $CD25^+CD45RA^+$ and $CD25^+CD45RO^+$ generated T_R cells to suppress was similar (Fig. 1*c*). In addition, expression of FoxP3 protein was only seen in $CD25^+$ cells and not $CD25^-$ from both the $CD45RA^+$ and $CD45RO^+$ generated T_R (Fig. 1*d*). These data demonstrate that $CD25^+$ T_R can be generated from both the naïve and memory $CD4^+$ T cell pools.

The generation cultures described above use an artificially strong form of activation for a brief period, in the form of an anti-CD3 mAb. To evaluate T_R generation under conditions more consistent with those found *in vivo*, in which APC and prolonged or repeated stimuli may be present, we studied T_R derived from T cells naïve to alloantigen (Fig. 2). $CD4^+CD25^-$ T cells were cultured for 10 days with allogeneic DC and then the $CD4^+$ T cells were separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ fractions by FACS and analyzed for suppressor function. $CD4^+CD25^+$ T cells from these cultures were able to suppress proliferation of freshly isolated autologous responder $CD4^+CD25^-$ cells activated with either the allogeneic DC or soluble anti-CD3/CD28 and autologous APC (Fig. 2*a*). Similar to results seen with our anti-CD3 generated T_R (27), suppression was lost when the responder cells were separated from generated $CD4^+CD25^+$ populations by a transwell (Fig. 2*a*), blockade of IL-10 or TGF-β did not alter the ability of the generated $CD4^+CD25^+$ T cells to suppress proliferation (data not shown), and $CD25^+$ cells sorted from the allogeneic DC activation culture expressed FoxP3 (Fig. 2*b*). In addition, allo-generated regulatory cells suppress with similar potency as CD3-generated regulatory cells from the same subject (Fig. 2*c*). Thus, $CD4^+CD25^+$ T_R that are contact-dependent, are IL-10- and TGF-β-independent, and express FoxP3 can be generated by activation of $CD4^+CD25^-$ T cells with allogeneic dendritic cells. Furthermore, when $CD4^+CD25^-CD45RA^+$ cells were activated by allogeneic DC to generate T_R, the resulting $CD25^+$ cells were regulatory (Fig. 2*d*), supporting our finding that naïve $CD4^+CD25^-$ T cells have the capacity to become T_R.

T_R Specific for a Foreign Antigen Can Be Isolated by HLA Class II Tetramers. To test whether an antigen-specific T_R recognizing a foreign epitope could be generated *in vitro*, we used a system suitable for analysis of T cell specificity using class II tetramers specific for a peptide from the influenza virus HA (306–319).

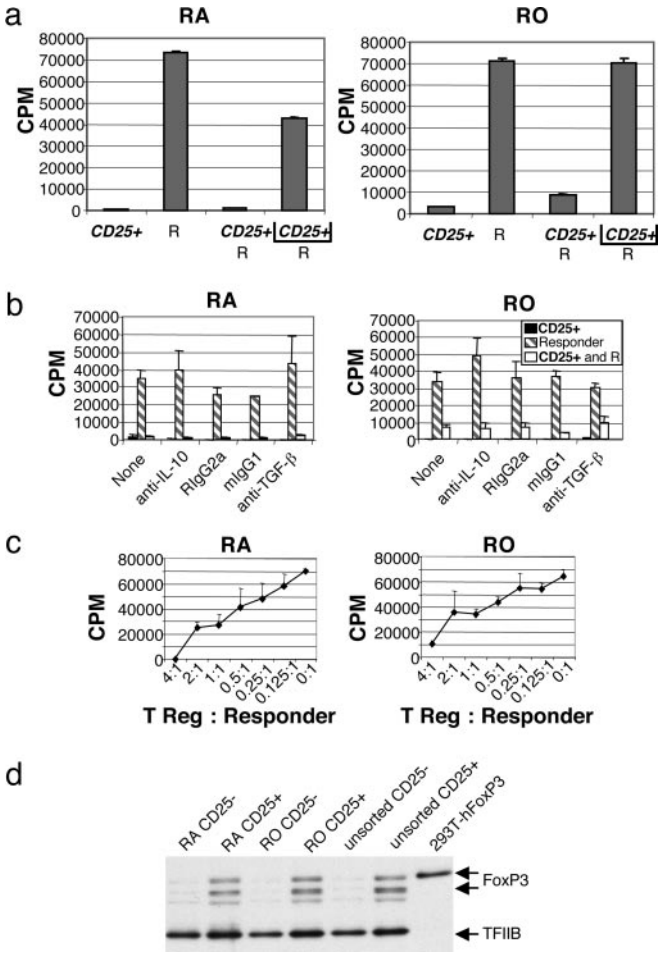

Fig. 1. $CD4^+CD25^+$ regulatory T cells can be induced by activation of memory or naïve human $CD4^+CD25^-$ PBMC with anti-CD3. (*a*) T_R were generated by activation of $CD4^+CD25^-CD45RA^+RO^-$ (naïve, *Left*) or $CD4^+CD25^-CD45RO^+RA^-$ (memory, *Right*) T cells on plate bound anti-CD3/soluble anti-CD28 overnight. After 10 days, $CD25^+$ cells from the generation culture were cultured with or without fresh $CD25^-$ cells from the same donor at a 1:1 ratio (*a* and *b*) or varying ratios (*c*) and stimulated with soluble anti-CD3/28. Cells were separated by a transwell (*a*) or cultured with the addition of 10 μg/ml anti-TGF-β, IL-10, or istotype control Ab (*b*). Cells designated in bold are from the generation culture and cells designated (R) are freshly isolated autologous $CD4^+CD25^-$ T cells. Data are presented as mean of triplicate cultures with error bars representing standard deviation. These data are representative of three experiments. (*d*) Western blot analysis of FoxP3 expression on day 10 of the generation culture for $CD4^+CD25^-CD45RA^+$, $CD4^+CD25^-CD45RO^+$, or unsorted $CD4^+CD25^-$ cells sorted into $CD25^-$ and $CD25^+$ subsets. Control cells were 293T cells transfected with a hFoxP3 cDNA clone.

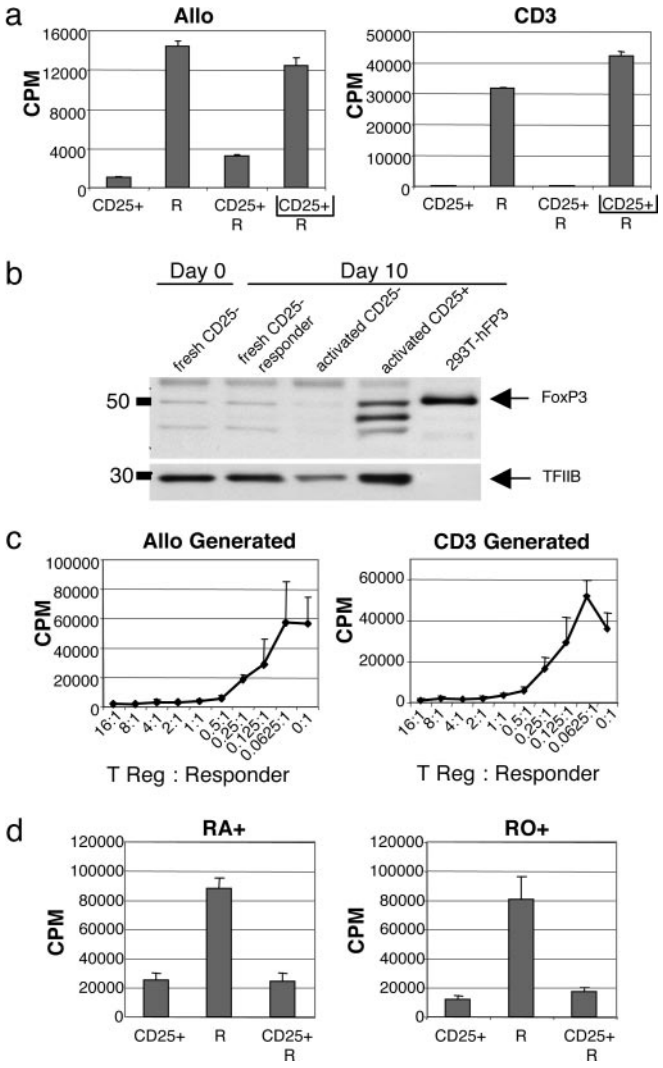

Fig. 2. Alloantigen-generated $CD4^+CD25^+$ regulatory cells suppress the proliferation of $CD25^-$ responder cells. (*a*) T_R were generated by activation of $CD4^+CD25^-$ T cells on allogeneic DC for 10 days and then sorted based on expression of CD25. Suppression by allo-generated $CD25^+$ cells when cultured with or without fresh $CD25^-$ responder (R) cells from the same donor or separated by a transwell (⊔) at a 1:1 ratio and stimulated with the original alloantigen or soluble anti-CD3/CD28 is shown. Results are presented as the mean of triplicates from one experiment with error bars representing standard deviations, and are representative of three experiments. (*b*) Western blot of FoxP3 expression in fresh $CD4^+CD25^-$ cells, $CD4^+CD25^-$ or $CD4^+CD25^+$ generated by activation on alloantigen, and 293T cells transfected with a hFoxP3 cDNA clone. (*c*) Allo-generated (*Left*) or CD3-generated (*Right*) $CD25^+$ cells (400,000–1,563 per well) were cultured at varying ratios with fresh $CD25^-$ responder T cells (25,000 per well) and stimulated with soluble anti-CD3/CD28. Results are presented as the mean of triplicates from one experiment with error bars representing standard deviations, and are representative of two experiments. (*d*) T_R were generated by activation of $CD4^+CD25^-CD45RA^+RO$- (naïve, *Left*) or $CD4^+CD25^-CD45RO^+RA^-$ (memory, *Right*) T cells on allogeneic DC for 10 days and then sorted based on expression of CD25. Suppression by allo-generated $CD25^+$ cells when cultured with fresh $CD25^-$ responder cells from the same donor at a 1:1 ratio and stimulated with the original alloantigen is shown.

A previous exposure to HA can occur via infection with *Haemophilus influenza* or through immunization, thus making this an antigen for which a recall response can be measured *in vitro*. In addition, MHC class II Tmrs specific for the peptide HA (306–319) in the context of DRB1*0401 have been used successfully to identify human antigen-specific T cells (26, 29, 30), allowing us to isolate T cells specific for HA (306–319) from culture. $CD4^+CD25^-$ T cells from three DRB1*0401 positive individuals, previously vaccinated for flu, were isolated and cultured for 10 days with irradiated, $CD4^+$ T cell-depleted autologous APC and 10 μg/ml HA (306–319) (Fig. 3*a*). HLA class II tetramers were used to isolate the HA-specific cells from the primary culture. Cells were stained with tetramer, CD4, CD25, and annexin V. The annexin V-negative cells were then sorted into three groups, those that were $CD4^+CD25^+$tetramer$^+$, $CD4^+CD25^+$tetramer$^-$, or $CD4^+CD25^-$. These three groups were then tested for their ability to suppress the proliferation of responder cells (freshly isolated, autologous $CD4^+CD25^-$ T cells) in response to HA

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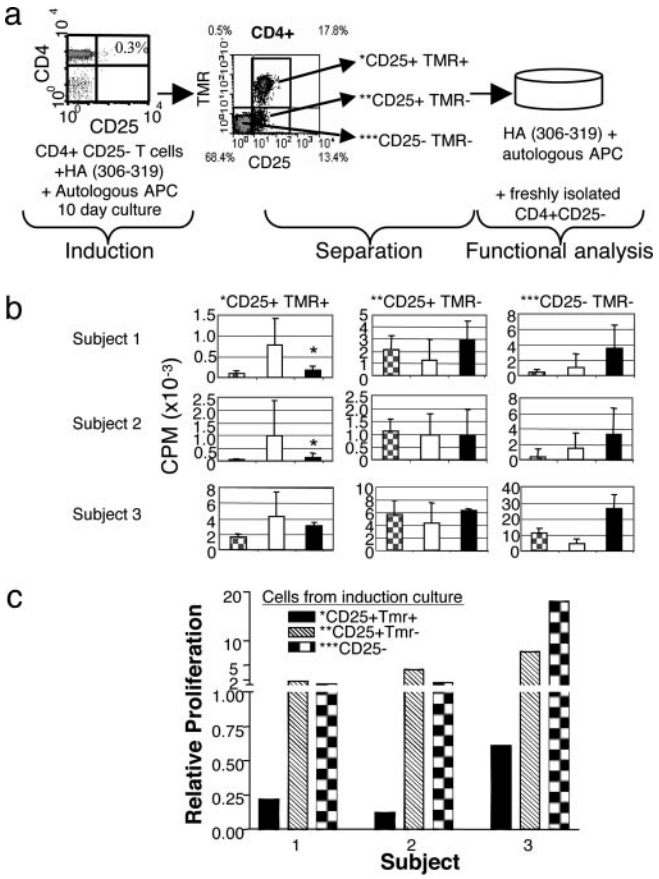

Fig. 3. Generation of and suppression by antigen-specific $CD4^+CD25^+$ regulatory cells. (*a*) Schematic of the culture system used to generate antigen-specific T_R. (*b*) $CD25^+TMR^+$ (*Left*), $CD25^+TMR^-$ (*Middle*), and $CD25^-TMR^-$ (*Right*) T cells were generated according to *a* from three separate individuals and cultured either alone (hatched bars) or together with fresh $CD4^+CD25^-$ responder cells from the same donor (filled bars). Proliferation of fresh $CD4^+CD25^-$ responder cells alone is shown by open bars. Data are presented as the mean of triplicates for each experiment with error bars representing standard deviation. These data are three separate experiments and are representative of five experiments. *, $CD25^+CD25^-$ proliferation is significantly different from fresh $CD25^-$ alone ($P < 0.5$). (*c*) Relative proliferation of responder cells cultured with generated $CD25^+Tmr^+$, $CD25^+Tmr^-$, or $CD25^-$ is shown for data presented in *b* with proliferation of fresh $CD25^-$ responder cells to HA (306–319) equal to 1 in this graph.

(306–319). The results from three separate subjects are shown in Fig. 3*b*. The proliferation of the responder cells was variable, likely because of low precursor frequency of HA-specific T cells in the responder population; however, suppression of proliferation in response to HA (306–319) was consistently seen with the $CD25^+HATmr^+$ population (Fig. 3 *b* and *c*). This HA (306–319)-specific suppression was seen with only the $CD25^+HATmr^+$, but not $CD4^+CD25^+HATmr^-$ or $CD4^+CD25^-$ cells taken from the same induction cultures. Therefore, T_R can be generated by activation with a foreign antigen and require activation with that cognate antigen for expression of suppressive activity.

To further test the specificity of the $CD4^+CD25^+Tmr^+$ cells, we examined whether the generated $CD4^+CD25^+HATmr^+$ cells could be activated by an unrelated antigen and mediate suppression in response to that antigen. Freshly isolated responder T cells were cultured with HA-derived T_R and tetanus toxoid as the stimulating antigen. The responder cells proliferated well when cultured with tetanus alone (Fig. 4), and no suppression was seen when HA-derived $CD25^+Tmr^+$

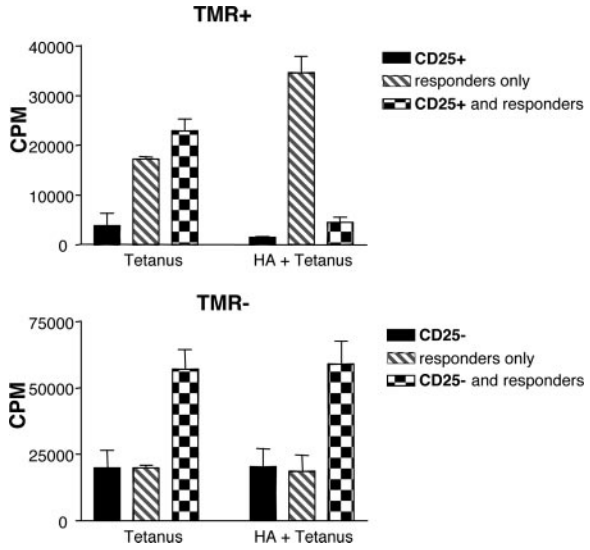

Fig. 4. Suppression by generated T_R requires specific antigen for activation; however, suppression is antigen nonspecific. T_R were generated by activation of $CD4^+CD25^-$ T cells on HA (306–319) for 10 days followed by sorting for tetramer binding. $CD25^+TMR^+$ (*Upper*) or $CD25^-TMR^-$ (*Lower*) cells from the generation culture were cultured with or without fresh $CD25^-$ responder cells from the same donor at a 1:1 ratio and stimulated with tetanus or HA plus tetanus. Data are presented as mean of triplicate cultures, with error bars representing standard deviation.

cells were added to these cultures. However, suppression was seen in cultures in which both tetanus and HA were mixed together as the stimulating antigens, demonstrating the requirement for specific activation of the HA-generated T_R. In addition, the almost complete suppression of proliferation in these cultures demonstrated the ability of HA-specific T_R to suppress T cells activated with another antigen. Thus, these HA-induced and tetramer isolated T_R require antigen specific activation, but, once activated, these cells are capable of nonspecific bystander suppression.

Similar to T_R directly isolated from the peripheral blood, suppression was reversed by the addition of IL-2 (data not shown) but not inhibited by the blockade of IL-10 or TGF-β (Fig. 5*a*). Examination of FoxP3 expression in these same experiments demonstrated that FoxP3 was present in the $CD4^+CD25^+HATmr^+$ and $CD4^+CD25^+Tmr^-$ cells, but not the $CD4^+CD25^-$ cells, from the HA-stimulated primary cultures (Fig. 5*b*). As described by Novak *et al.* (29), this type of culture produces both antigen-specific Tmr^+ T cells and bystander activated T cells that are Tmr^-. To further test the suppressive function of the "bystander" $CD4^+CD25^+HATmr^-$ cells, these cells were isolated and cultured with fresh $CD4^+CD25^-$ responders, using a nonspecific (anti-CD3/irradiated APC) stimulus. Consistent with their inability to bind the HATmr, these cells did not suppress in cultures activated with HA, but were able to suppress when activated with anti-CD3 and irradiated APC (Fig. 5*c*). Thus, the generation and isolation of antigen-specific T_R required separation of specifically activated induced T_R, in this case using HLA class II tetramers.

Discussion

Immune responses are controlled by at least three classes of regulatory T cells: $CD4^+CD25^+$ T_R, T helper 3 (Th3), and T regulatory type 1 (Tr1) cells. In mice, $CD4^+CD25^+(T_R)$ are thought to arise in the thymus, express the *forkhead*/winged helix transcription factor FoxP3, and suppress the *in vitro* proliferation and cytokine production of effector T cells in a cell contact-

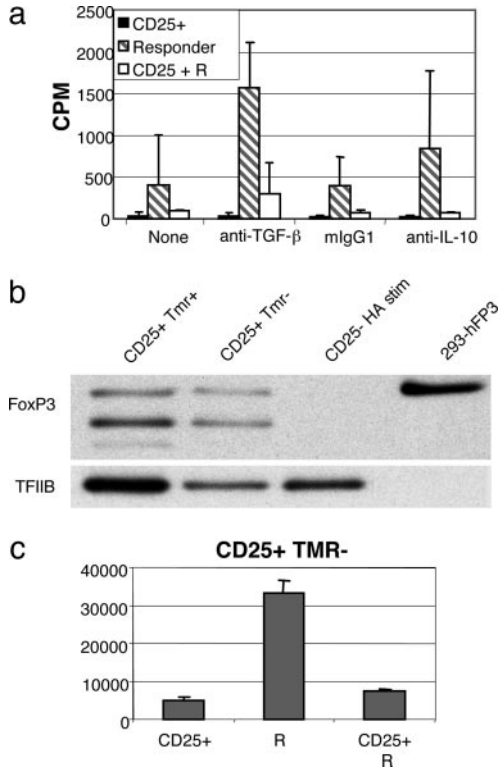

Fig. 5. $CD4^+CD25^+$ T_R generated by specific antigen are IL-10- and TGF-β-independent and express FoxP3. (*a*) HA-generated regulatory cells were cultured in a HA-stimulated suppression assay at a 1:1 ratio with responder $CD25^-$ in the presence of 10 μg/ml anti-IL-10, anti-TGF-β, or an isotype control Ab. These data are representative of two experiments. (*b*) Western blot of FoxP3 expression in cells from the generation culture or 293T cells transfected with hFoxP3. (*c*) HA-generated $CD25^+TMR^-$ cells are cultured alone or with fresh $CD25^-$ responder cells from the same donor at a 1:1 ratio and stimulated with soluble anti-CD3/CD28. Data are presented as mean of triplicate cultures from one experiment with error bars representing standard deviation and are representative of five experiments.

dependent manner (31–34). Tr1 and Th3 cells are generated in the periphery during immune responses and elicit their suppression via cytokine-dependent mechanisms, IL-10 for Tr1 cells and TGF-β for Th3 cells (35, 36). Based on these data, a model has been proposed suggesting that the $CD4^+CD25^+$ T_R cells represent natural regulatory T cells, whereas the Tr1 and Th3 cells represent acquired regulatory T cells (37). In this model, T_R cells are involved in protection against self-responses, whereas the acquired subsets can respond to both self and foreign antigens. More recently, this distinction has become less clear, as studies have demonstrated that FoxP3 expression and T_R function can be induced in $CD4^+CD25^-$ T cells under a variety of conditions (17, 21–23, 38).

These same regulatory T cell subsets have been identified in humans. It has been shown that $CD4^+CD25^+$ T_R isolated directly from human peripheral blood express FoxP3 and suppress T cell proliferation in a cell-contact-dependent, cytokine-independent manner when studied *in vitro* (27, 39–41). However, the source of human $CD4^+CD25^+$ T_R cells, whether the thymus, the periphery, or both, is not known. We have recently shown that T_R cells displaying the properties of ''natural'' $CD4^+CD25^+T_R$ (contact-dependent, IL-10- and TGF-β-independent suppression of T cells) can be generated *in vitro* from nonregulatory $CD4^+CD25^-$ T cells (27). We now demonstrate that such T_R arise during antigen-stimulated T cell expansions, and that a set of antigen-specific T_R can be isolated that, when triggered with cognate antigen, initiate bystander suppression. In addition, we show that such effector T_R cells can be generated from both naïve and memory $CD4^+CD25^-$ T cell precursors and are capable of suppressing other T cells in a cell-contact-dependent manner.

The *in vitro* generation of T cells with a T_R phenotype has been described by other groups, using a variety of conditions. For example, $CD4^+CD25^+$ T_R specific for alloantigen can be expanded from the blood of patients receiving allogeneic bone marrow transplantation (42). In addition, T_R specific for tumor antigens have been cloned and shown to have properties similar to $CD4^+CD25^+$ T_R cells. These clones, like the HA-specific T_R described above, required activation with cognate antigen for elaboration of regulatory function, but once activated, suppression was antigen nonspecific (9, 10). Finally, there is increasing evidence that T_R can be generated in the periphery of mice (14, 17, 23, 43), and a recent report describes the induction of antigen-specific $CD4^+CD25^+$ T_R from $CD4^+CD25^-$ T cells *in vivo* (44).

Despite these data showing the ability to generate T_R cells *in vitro*, the signals that control this differentiation remain unknown. TGF-β has been implicated in both the *in vitro* generation and function of T_R-like cells in several systems (21, 22, 45). In the system described here, neutralization of TGF-β had no effect on the suppression exhibited by *in vitro*-generated antigen-specific T_R (Fig. 5*a*). In addition, exogenous TGF-β was not added to the generation cultures, and neutralization of TGF-β had no effect during the generation of T_R with anti-CD3 (data not shown). Similarly, blockade of IL-10 had no effect on either the generation or function of the *in vitro*-derived T_R. Thus, the T_R we have identified in these studies do not appear to depend on IL-10 or TGF-β for their differentiation or function.

Our ability to isolate T_R from *in vitro* cultures similar to those conditions traditionally used to expand antigen specific T cells raises the question of how long term T cell cultures can be cultivated by using such a system. It is important to note that, in this system, only those T cells that remain $CD25^+$ 10 days after activation are $FoxP3^+$ and have suppressive function, whereas in typical activation cultures, the great majority of T cells present after 10 days have become $CD25^-$ and that among the $CD25^+$ group, not all cells are regulatory. Thus, the *de novo* generated T_R population can be viewed as a small, dedicated subset of activated cells persisting within the memory T cell response. It is also noteworthy that traditional restimulation of human T cell lines and clones are done with the addition of exogenous IL-2. The *in vitro* suppression assay, on the other hand, measures proliferation in the absence of IL-2 and suppression is in fact abrogated by IL-2; thus, standard culture conditions used to grow antigen-specific T cells are not likely to demonstrate the presence of this type of T_R cell.

These data suggest a model whereby T_R cells are generated during an immune response in humans, and are involved in controlling the spread of the response. In this model, T_R cells are generated either after activation of naïve cells or from effector cells later in the response. These T_R cells are then responsible for controlling the spread of the response through suppression of both responder/effector cells as well as bystander activated cells. The origin of generated T_R is unknown; we cannot rule out the possibility that these cells derive from rare $CD25^-FoxP3^+$ cells that become $CD25^+FoxP3^+$ upon activation. We have seen faint bands for FoxP3 expression by Western blot in $CD25^-$ cells along with other groups (34, 46). The fate of generated T_R is not yet clear; these cells may be short lived, or they may persist as part of the memory population. This will be an important factor with clinical implications, because human $CD4^+CD25^+$ T_R cells that are generated in the periphery to foreign and self-antigens very

likely are important for the down-regulation of immune responses to those antigens. It has been demonstrated in transgenic mouse models that the transfer of T_R specific for pancreatic antigens can prevent and cure autoimmune diabetes (24). An understanding of this process and how to enhance or inhibit these T_R in humans may prove useful in therapeutic approaches both for autoimmunity and transplantation.

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